Final Report: Optimization and Clearance Studies of a New Hormone-Based Spawning Induction Technology for Aquacultured Finfish.

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Abstract

This research has further characterized and optimized a GnRH-based spawning induction technology for striped bass and related species, using sustained-release, biodegradable GnRHa delivery systems. We demonstrated that D-Arg^6-cGnRH-II (an analog of a common perciform GnRH) is equally potent compared to the D-Ala^6-mGnRH agonist (which is currently used in culture situations). Doses for acute administration and analog effectiveness in eliciting *in vivo* gonadotropin release have also been established. Finally, our data demonstrated rapid and satisfactory clearance rates of the GnRHa from fish tissues. This research was conducted in view of facilitating regulatory approval of the GnRH-based technology and its large-scale application in the aquaculture industry.

Executive Summary

This report summarizes research on a spawning induction technology for commercial fish species. Data included within this report suggests that an analog of chicken-II gonadotropin releasing hormone, a type of native fish gonadotropin, may be the best candidate for use in future spawning induction technologies. In addition to physiological data suggesting that it is an effective gonadotropin releaser, the analog may be a better candidate for regulatory approval of technology. Additional experimental data is expected to establish the receptor-binding properties of this promising analog.

Purpose

One of the major bottlenecks to the development and intensification of finfish aquaculture is the fact that the majority of commercially important farmed fish exhibit different degrees of reproductive disorders when raised in captivity. Many farmed fish do not ovulate or spawn spontaneously in confinement. As a result, commercial farming of several fish species completely depend on the rather unreliable captures of gravid broodstocks on their spawning grounds or the collection of juveniles in the wild. For example, the hybrid striped bass industry in the U.S. still relies to a large extent on eggs produced from broodstock captured on their spawning ground throughout a relatively short season. Other fish, such as many salmonid species, do ovulate spontaneously in captivity. However, ovulation and spawning in such cases are highly non-synchronized, which results in unpredictable seed supply. Yet another reproductive problem is found in the males of several species that produce very small volumes of sperm when raised in captivity.

The need to reliably provide finfish eggs and juveniles resulted in the development of captive broodstock programs. In order to induce captive fish to spawn in a predictable manner, hormone-based treatments have been developed. For many years, the most frequently used techniques involved the injection to both females and males of different preparations of gonadotropins. The two most widely used gonadotropins have been pituitary extracts (hypophysation) and human chorionic gonadotropin (hCG). Although successful, the use of gonadotropins faces some major limitations that will prohibit its acceptance as a generic spawning-induction technology. Among other problems, such extracts, produced from pituitary homogenates, are potential carriers of diseases, mainly viral in nature. Finally, because fish

gonadotropins are highly species specific, pituitary extracts front a given fish are of a restricted utility for other species.

Our laboratory has developed a controlled release implant for delivering gonadotropin-releasing hormone; analog (GnRHa) and, consequently, inducing captive spawning. However, before the GnRHa controlled release spawning-induction technology can be largely applied to the industry, it will have to be fine-tuned, optimized and, ultimately, gain regulatory approval. This proposal aims at providing information that will facilitate upscaling this technology for use in commercial broodstock management programs in finfish aquaculture. This, in turn, will enable a reliable, predictable, and continuous supply of finfish seeds to all interested growers. We will use two commercially important *Morone* species (striped and white bass) as our research models and will identify the most potent GnRH analog to be used as well as its optimal biodegradable delivery system. We will determine the optimal doses to be injected and study metabolic clearance rates of the injected hormone from blood and tissues of the treated fish. Finally, we will study the possible presence of GnRHa in the spawned eggs and offspring and its metabolic clearance.

The main objective of this proposal is to optimize a GnRH-based spawning induction technology for striped bass and related species, using sustained-release, biodegradable GnRHa delivery systems, and to study the clearance rates of the GnRHa from fish tissues. This is being done in view of facilitating regulatory approval of the GnRH-based technology and its large-scale application in the aquaculture industry.

Approach

The project objectives arc being met through the following steps:

1. Evaluate the potency of analogs of the novel GnRH (i.e., sbGnRH) found in perciform fish and select the most potent one. Analogs of sbGnRH, the GnRH form implicated as the relevant one to the processes of ovulation and spawning, will be synthesized and injected to female striped bass. Their potency in inducing GtH-II secretion will be compared to the currently used GnRHa. The most potent analog will be identified and selected.
2. Optimize the GnRHa delivery system. The most potent GnRHa will be incorporated into two biodegradable polymeric delivery systems. They will be injected in decreasing doses into female and male striped and white bass. The most potent system and efficient dose, in terms of inducing ovulation, spawning, and spermiation, will be identified.
3. Study the metabolic clearance rates of the GnRHa and its metabolites from fish tissues. The GnRHa delivery systems will be injected into the fish. At different time intervals thereafter, levels of the GnRHa and its metabolites will be determined in the blood and various tissues of the fish.

All work was conducted by the individuals under the supervision of Dr. Yonathan Zohar at the University of Maryland Biotechnology Institute's Center of Marine Biotechnology.

Summary of Findings

The cumulative progress on each of the individual objectives is noted in detail below.

Findings from Objective l

We previously evaluated the gonadotropin (GtH) II-releasing activity of various new agonists of gonadotropin-releasing hormone (GnRHa), at two doses for each agonist, in the striped bass *(Morone saxatilis),* and compared them to [des-Gly^{10}, D-Ala^{6}, Pro(-Net]-mammalian (m) GnRH (D-Ala^{6}-mGnRH), which is one of the more potent and widely-used agonists. Briefly, novel GnRH analogs were synthesized by a [des-Gly^{10}, D-amino acid^{6}, Pro^{9}]-substitution of the primary structure of two of the native GnRH forms identified in striped bass, namely seabream (sb) GnRH ([Ser^{8}]-GnRH) and chicken (c) GnRH-II ([His^{5}-Trp^{7}-Gln^{8}]-GnRH). At a dose of 50 : g/kg body weight (BW), all GnRH analogs induced significant increases in plasma GtH II levels compared to salineinjected controls (P# 0.05), with D-Ala^{6}-mGnRH and D-Arg^{6}-cGnRH-II inducing the highest increase, reaching mean (± s.e.m) maximal GtH II concentrations of 35.1± 4.7 ng/ml. At a dose of 5 : g/kg BW, the D-Trp^{6}-sbGnRH did not elevate plasma GtH II, whereas both D-Ala^{6}-sbGnRH and D-Ala^{6}-cGnRH-II induced significant increases (P# 0.05 and P# 0.01, respectively). Similar to the higher dose, females injected with either D-Ala^{6}-mGnRH or D-Arg6-cGnRH-II had the highest plasma GtH-II levels, which were significantly higher compared to any of the other GnRHa-treated groups (P# 0.01). This interesting but surprising result indicates that analogs of a striped bass GnRH form, which was not believed to be the most relevant for the regulation of ovulation and spawning, are actually the most potent.

Since, at a dose of 5 : g/kg BW, there were two GnRHa with similar GtH-II-releasing potencies, a third experiment using a lower dose (1 : g/kg BW) was carried out in February 1999 in an effort to identify potential differences between the two agonists. However, D-Ala^{6}-mGnRH and D-Arg^{6}-cGnRH-1I demonstrated the same GtH II-releasing activity also when injected at a 1 : g/kg BW dose.

It is now apparent in light of the data that D-Arg^{6}-cGnRH-II is equally potent compared to the D-Ala^{6}-mGnRH agonist. However, the fact that cGnRH-II has an ubiquitous distribution across the vertebrate species led us to speculate that analogs based on cGnRH-II may have a generic application. Recent data from our lab have also established a correlation between gonadal development and the levels of native cGnRH-11 and sbGnRH in the brain and pituitary. It is also believed that use of cGnRH-II agonist in spawning induction technology will have a wider acceptance by the consumer and that obtaining approval for its commercial use can be expedited, mainly because cGnRH-II is a native GnRH form present in the fish. The results presented here on the potency of cGnRH-II agonist to release GtH-II in striped bass is based on its acute administration.

Currently, we are in the process of incorporating D-Arg^{6}-cGnRH-II in the controlled-release delivery system in order to obtain a sustained release pattern of this agonist *in vivo.* However, as noted above, the most potent GnRH agonist (cGnRH-II) is not yet characterized to a point which would justify incorporation into an *in vivo* delivery system. We are taking steps to characterize the agonist to our satisfaction, prior to finalizing the incorporation. Most recently, we have also cloned the GnRH receptor from striped bass (see subsequent paragraphs), and that is being used to compare the binding affinities of the tested agonists, with the goal being to further improve the spawning induction technology.

We have cloned the full length cDNA for striped bass pituitary GnRH-R. Using 5' and 3', specific primers in the UTR region of the complete GnRH-R cDNA the coding region were amplified and then cloned in a sense and antisense direction (for control) in an eukaryotic expression vector pcDNA 3.1 under the cytomegalo virus (CMV) promotor at Eco RV site. pcDNA3.1/zeo is an expression vector that has been designed for high-level constitutive expression in a variety of cell line. The striped bass GnRH-R cDNAs isolated have been ligated in this vector in sense and antisense direction following the standard ligation procedures. Positive clones have been selected based on the sequencing data and restriction enzyme analysis.

CHSE-214 fish cells and COS-7 cells have been obtained from ATCC and are being cultured routinely in our laboratory. Preliminary transformation with the pcDNA-GnRH-R (both sense and antisense directions have been done) was carried out, and currently we are selecting the cell-line which are expressing fish GnRH-R. We have recently generated a stable fish cell line by zeocin selection. Binding affinity studies with the purified recombinant receptor are expected to elucidate the potency, and the corresponding potential for commercial application, of cGnRH-II agonists.

Findings from Objective 2:

As noted above, our results clearly implicate cGnRH-II agonist as equally potent in eliciting the GtH-II response. Therefore, we have chosen to incorporate D-Arg^6-cGnRH-II into our delivery systems. However, because of the significant expense of purchasing and incorporating the hormone, we are trying to obtain more definitive data from our receptor binding studies with D-Ala^6-mGnRH and D-Arg^6-cGnRH-II before we start our *in vivo* field trial studies in striped bass and other species. Pending satisfactory results for the cGnRH-II agonist in our recombinant receptor binding assay, we will proceed with the scheduled incorporation and in vivo characterization of the cGnRH-II agonist controlled release system.

Findings from Objective 3:

As noted in several previous progress reports, GnRHa levels in GnRHa-induced females have been undetectable in all tissues tested (blood, ovaries, eggs, etc) at all time points tested.

Need for additional research:

As noted above, we are taking steps to better characterize the agonist, prior to finalizing the incorporation into a controlled release device. To this end, we are developing a receptor based system using the cloned GnRH receptor from striped bass and a stable fish cellline expressing the receptor. This system will allow us to compare the binding affinities of the tested agonists, with the goal being to further improve the spawning induction technology.

Evaluation:

All project goals were obtained, with the notable exception of incorporation of the native GnRH analog into a controlled release delivery system. This is an expensive and time-consuming process. As noted above, our laboratory determined that, based on our findings during this project, additional characterization of the best prospective analog(s) should be undertaken prior to the incorporation step.

A significant but very important supplemental assay (i.e., the receptor-based assay noted above) is being developed to better achieve the critical elements of objective 2 of this project.

Results from this project have been and will be disseminated to academic and industrial personnel at the 6th International Symposium on Reproductive Physiology of Fish in Bergen, Norway, July 4-9, 1999 and the 4th International Symposium on Fish Endocrinology in Seattle, Washington, July 31-August 3, 2000.